REMARKS

Claims 1, 2, 4-7, 10, 14, 15, 18, and 27-32 remain pending in the case. Claims 1, 2, 6, and 7 are amended above. Claims 10 and 18 are withdrawn as drawn to unelected inventions. Claims 1, 2, 4-7, 14, 15, and 27-32 are under examination. All claims under examination stand rejected for lack of enablement under 35 USC 112, paragraph 1.

Interview summary

Applicant wishes to express its appreciation to the Examiner for granting a telephonic interview ("the Interview") with the undersigned on September 23, 2010. The above amendment of claims 1, 2, 6, and 7 was discussed; the Examiner agreed that it would be entered after final, without the necessity to file an RCE. Also discussed were the scope of the claims and the present rejection for lack of enablement. The undersigned explained that the method of claim 1 is drawn to a screening method for identifying a light chain that is able to pair with both a first Fd, to bind to a first antigen, and a second Fd, to bind to a second antigen. Following that explanation, the Examiner clarified that the enablement rejection is now based upon a concern that a light chain selected in accordance with the claimed invention might be merely "cross-reactive" as opposed to binding the antigens "specifically." This new concern, as well as the concerns expressed in the Final Office action dated March 17, 2010 (the "Final Office Action"), are discussed below.

In the Interview, the Examiner did not elaborate on what she saw as the distinction between "cross-reactive" binding and "specific" binding. The Interview Summary dated September 28, 2010, adds a little explanation: "**The question is really whether the selected light chain, being successfully paired with any Fd to a different antigen, is truly representative of specific (cross-reactive) versus exclusive binding for any of the different and subsequent tested antigens?"** In the quoted sentence, the term "cross-reactive" appears to be used as a further definition of "specific," and both are said to be "versus" (i.e., distinct from) "exclusive binding." However, the next sentence of the Interview Summary sorts out these terms differently, using the term "cross-reactive" to mean something that is distinct from "specific" and

saying that the term "specific" is "within the meaning of exclusive." That sentence reads, "**There are no working examples to demonstrate the affinity of the selected rounds of antibodies to determine whether the binding is cross-reactive or specific within the meaning of exclusive**." Applicant assumes these inconsistencies to be inadvertent, and interprets the Interview Summary as intending to say that "cross-reactive" binding is different from "specific" binding, and the term "specific" means "exclusive," and that one can distinguish between "cross-reactive" binding and "specific" or "exclusive" binding by determining affinity of binding. If this is an incorrect interpretation, the Examiner is asked to clarify what was meant.

The Examiner's concern (as Applicant understands it) is focused on whether a light chain identified in the method of claim 1 (i.e., a light chain that can functionally pair with either of two different Fd) is "specifically" binding to each of the two antigens when paired with the cognate Fd, or alternatively is merely "cross-reacting" with one or both of the antigens.

First, Applicant points out that claim 1 does not require determination of just how the light chain itself is interacting with the antigen. In fact, no step of claim 1 involves any sort of evaluation of antigen binding by the light chain alone. Rather, the two selection steps of claim 1 both involve phage display of two-chain (i.e., Fd plus light chain) antibody fragments and selection of two-chain antibody fragments that bind specifically. In a two-chain antibody fragment, the antigen specificity can be contributed solely by the Fd CDRs or by both the Fd and light chain CDRs. Whether the light chain contributes to the antigen binding of each of the two-chain antibody fragments in an epitope-specific manner or instead is epitope-generic in one or both of the two-chain fragments (e.g., by simply stabilizing the Fd's shape and thereby enhancing the affinity of the Fd's binding) is not an aspect of the invention, and should not be a concern. The only light chains that will be expressed in the phage libraries selected in step (e) of claim 1 will be those that are able to pair successfully with both the first and second Fd to permit binding of the first Fd to the first antigen and the second Fd to the second antigen. The screen can be adjusted as needed to require higher or lower levels of affinity: e.g., if there are few hits, then one can reduce the stringency of the screen to pick up more hits, e.g., by panning for a longer time or rinsing a shorter time; if there are many hits, then the stringency can be increased

to raise the threshold affinity--whatever works best to identify a usable number of hits. Thus, the Examiner's concern that there are "no working examples to demonstrate the affinity of the selected rounds of antibodies to determine whether the binding is cross-reactive or specific within the meaning of exclusive" is entirely irrelevant to the question of whether one of ordinary skill in the art can carry out the claimed methods.

In case the Examiner's concern about the absence of working examples is in part due to a fear that light chains cannot functionally associate with more than one Fd (i.e., cannot be "promiscuous"), Applicant notes that no evidence to support such a fear has been made of record. Under U.S. law, a specification must be taken to be enabling, absent evidence or reasoning establishing that one of ordinary skill in the art would not believe the disclosure. See, e.g., *In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971); and *In re Wright*, 999 F.2d 1557, 27 USPQ2d 1510 (Fed. Cir. 1993). In fact, the existence of light chains that can pair with different Fd to promote high-affinity binding of the Fd to their cognate antigens is well accepted in the art. See, e.g., Merchant et al., Nature Biotechnology 16:677-681 (1998), attached hereto as Exhibit A. Merchant et al. describes a bispecific IgG constructed of two different heavy chains and two identical light chains. (The light chain was identified by a technique quite different from the presently claimed technique, including use of recombinant scFv phage libraries "that have extensive H chain repertoires." See, Merchant et al., page 677, left column, sentence immediately prior to "Results" section.) According to Merchant et al. at page 680, right column, second full paragraph, "**These high affinities presumably reflect the fact that the major determinants contributing to the specificity and energetics of antigen binding reside on the H chain for these phage-derived antibodies**." Accordingly, there is no question that those of ordinary skill in the art would have expected it to be possible to identify light chains that can pair productively with two different Fd—i.e., permit the Fd to bind to its cognate antigen.

The Interview Summary cites MPEP 2138.05 and *Birmingham v. Randall* in support of the enablement rejection, apparently in the belief that this case and the MPEP cite establish that there is a requirement for working examples proving that the claimed method works. Applicant submits that neither *Birmingham v. Randall* nor MPEP 2138.05 is on point, as both concern the level of proof necessary to establish actual reduction to practice in the context of a priority of

invention dispute in an interference. This is not an interference proceeding, and neither priority of invention nor reduction to practice is at issue in the present case. More relevant to the examination context is MPEP 2164.02, which discusses working examples in the context of enablement (rather than priority of invention) and makes it clear that a specification need not contain a working example in order to satisfy the enablement requirement: **"The specification need not contain an example if the invention is otherwise disclosed in such a manner that one skilled in the art will be able to practice it without an undue amount of experimentation.** ***In re Borkowski*****, 422 F.2d 904, 908, 164 USPQ 642, 645 (CCPA 1970)."** In the present case, the specification provides ample disclosure to permit one of ordinary skill in the art to practice the claimed method without an undue amount of experimentation, using standard techniques well known in the art.

In view of the above, Applicant submits that the enablement concerns expressed during the Interview have been overcome.

It was Applicant's impression, following the Interview, that the above-discussed new basis for the enablement rejection expressed for the first time in the Interview had replaced the rationale for the rejection as stated in the Final Office Action. In case this impression was mistaken, Applicant will briefly address the prior rationale for the rejection as distilled in the "Response to Arguments" section of the Final Office Action at pages 5-7.

> The Final Office Action says at pages 5-6 that
>
> the examiner concludes that the claims recite elements that are not supported by the specification that would permit an operable method. For example the specification only teaches the following: *[citing paragraphs [0062] and [0068] of the published US application*]....This above recited paragraphs and the figures are the extent of written support and enablement for the instant claimed method.

This passage is not understood. The specification obviously does not "only teach" what is in paragraphs [0062] and [0068]. Relevant teachings can be found throughout the 76 paragraphs of the specification. See in particular paragraphs [0057]-[0067] and [0070]-[0073], which are discussed further below. It is not clear why the Examiner believes that only paragraphs [0062] and [0068] and the figures are "the extent of written support and enablement." Indeed, paragraph [0068] is about eukaryotic cells, so is less pertinent than much of the rest of the specification not referenced in the above-quoted passage.

The Final Office Action states at page 7, "The specification does not teach a vector encoding a secretion signal linked to the Fd to be used in combination with a vector encoding the light chain, a bacterial signal sequence and a phage membrane anchor where both are to be used for secretion in prokaryotic cells." Applicant disagrees. Paragraph [0057] teaches host cells that secrete antibody heavy chains (with Fd "particularly preferred"). Paragraph [0058] says that the genes encoding heavy chains are put onto vectors suitable for expression in the host cells, and says the host cells can be bacteria. Paragraphs [0059]-[0060] provide further detail how the vectors would be constructed and introduced into host cells. Paragraph [0064] describes secretion signal sequences, including the pel1B secretion signal that is commonly used in bacteria. *Thus, the specification clearly describes how one can make the first and second plurality of bacterial host cells specified in parts (a) and (d) of claim 1.* Paragraphs [0061]-[0062] describe phage libraries expressing light chains as fusion proteins containing a prokaryotic cell secretion signal domain, a light chain, and a phage membrane anchor domain. Paragraphs [0063]-[0064] provide further detail about the phage membrane anchor domain and secretion signal domain, respectively. Paragraphs [0065]-[0066] discuss phage particles generally. *Thus, the specification clearly describes, in substantial detail, how one could make the antibody light chain phage library specified in part (b) of claim 1.* Paragraphs [0066]-[0067], supplemented by Fig. 1, describe introduction of the light chain phage library into the host cells secreting heavy chains (or Fd) and presentation of two-chain antibody fragments (Fd plus light chain) on the surface of the phage particles, where the two-chain antibody fragments are available for screening. *Thus, the specification clearly describes how one can carry out steps (b) and (d) of claim 1.* Paragraph [0070] explains selection of the phage-presented antibodies based on binding to the first antigen, e.g., by panning, *thereby illustrating how the selection steps of claim 1 can be accomplished.* Paragraph [0071] discusses introducing the initially selected phage library (expressing the initially selected subset of light chains) into host cells secreting a second Fd specific for a second antigen, so that phage presenting two-chain antibody fragments containing the second Fd and a light chain are produced. *This provides guidance as to how step (d) of claim 1 could be accomplished.* Paragraph [0072] describes screening those phage to

select two-chain antibody fragments that bind to the second antigen. *This corresponds to step (e) of claim 1.* Paragraph [0073] points out that one can conduct multiple rounds of screening and introduction of the phage libraries into host cells, *illustrating the method as claimed in dependent claim 5.* Given all of this disclosure, and the fact that those of ordinary skill in the art at the time of the invention are accustomed to working with phage libraries, including antibody display libraries, and working with bacteria that secrete recombinant polypeptides, it is unclear to Applicant why the Final Office Action questioned enablement at all. It is logical to conclude that the claimed method will successfully select for "promiscuous" light chains capable of pairing both with a first Fd to bind to a first antigen (since the light chains were selected in association with the first Fd in the first selection step) and with a second Fd to bind to a second antigen (since the light chains were also selected in association with the second Fd in the second selection step) . Accordingly, there would seem to be no enablement issue.

Applicant reads the language at page 7 of the Final Office Action asserting "**The specification does not teach a vector encoding a secretion signal linked to the Fd…**" as possibly meaning the Examiner believes there is not adequate written description support for the "wherein" clauses of claim 1, parts (a) and (d), i.e., the clauses specifying that "the bacterial host cells contain[] DNA encoding the…Fd linked to a bacterial secretion signal peptide," and the corresponding "wherein" clauses of claims 2, 6, and 7. Although there certainly is implicit support for this clause in the specification (see, e.g., paragraphs [0057]- [0059] discussing bacterial host cells expressing vectors encoding heavy chains such as Fd and secreting the Fd, and paragraph [0064] discussing secretion signal peptides), the exact words of the clause do not appear *ipsis verbis* in the specification. Rather than argue the point, Applicant has simply deleted the clause from the claims. It is unnecessary verbiage anyway, as one of ordinary skill in the art would understand that the claim language "bacterial host cells secreting a Fd" that remains in the claims implies that there is a mechanism causing the bacterial host cells to secrete the Fd, and that the standard way to do that is by including in the host cells a DNA that encodes the Fd linked to a secretion signal.

CONCLUSION

It is believed that all issues have been addressed. Withdrawal of the rejection and allowance of the claims are respectfully requested. If any issues remain, the Examiner is asked to telephone the undersigned to discuss, in order to expedite resolution.

Please extend the time for filing an appeal brief for three months, to February 16, 2011. The extension fee in the amount of $1,110.00 is being paid concurrently herewith on the Electronic Filing System (EFS) by way of Deposit Account authorization. Apply any other charges or credits to deposit account 06-1050, referencing Attorney Docket No. 14875-0148US1.

Respectfully submitted,

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An efficient route to human bispecific IgG

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Production of bispecific IgG (BsIgG) by coexpressing two different antibodies is inefficient due to unwanted pairings of the component heavy and light chains. To overcome this problem, heavy chains were remodeled for heterodimerization using engineered disulfide bonds in combination with previously identified "knobs-into-holes" mutations. One of the variants, S354C:T366W/Y349′C:T366′S:L368′A:Y407′V, gave near quantitative (~95%) heterodimerization. Light chain mispairing was circumvented by using an identical light chain for each arm of the BsIgG. Antibodies with identical light chains that bind to different antigens were identified from an scFv phage library with a very restricted light chain repertoire for the majority (50/55) of antigen pairs tested. A BsIgG capable of simultaneously binding to the human receptors HER3 and cMpl was prepared by coexpressing the common light chain and corresponding remodeled heavy chains followed by protein A chromatography. The engineered heavy chains retain their ability to support antibody-dependent cell-mediated cytotoxicity as demonstrated with an anti-HER2 antibody.

Keywords: applied immunology, antibody engineering, phage display

The therapeutic potential of bispecific antibodies has been minimally explored due to inefficient production methods. Robust recombinant routes have been developed for bispecific antibody fragments[1-3] but not for bispecific IgG (BsIgG). Many applications of bispecific antibodies will require an IgG as this format provides an Fc region, comprising C_H2 and C_H3 domains, that can confer long serum half-life and support secondary immune functions.

The method of choice for preparing BsIgG has been the coexpression of two different IgGs in hybrid hybridomas[4]. IgG coexpression may produce up to 10 heavy (H) and light (L) chain pairings[5], thereby compromising the yield of BsIgG and often imposing major purification challenges. IgG H chains form homodimers, as well as the desired heterodimers, and L chains are prone to pair with noncognate as well as their cognate H chains.

We describe a method for the construction of human BsIgG that eliminates L chain mispairing and greatly diminishes H chain mispairing (Fig. 1). Antibody H chains have previously been remodeled for heterodimerization by rational design[6] and optimization by phage[7]. We have investigated the ability of engineered inter-C_H3 domain disulfide bonds to further enhance H chain heterodimerization. Disulfide bonds were also evaluated as an analytical tool to distinguish heterodimers from homodimers by SDS-PAGE as engineered disulfide bonds can increase the electrophoretic mobility of proteins when analyzed by SDS-PAGE under nonreducing conditions[8-10]. The L chain mispairing problem was circumvented by constructing a BsIgG from antibodies that use identical L chains. Such antibodies are routinely isolated from phage libraries that have extensive H chain repertoires and have unique[11] or very few[12] L chain sequences.

Results

Design of C_H3 variants. Antibody H chains were remodeled for heterodimerization using engineered disulfide bonds both on their own and in combination with sterically complementary, knobs-into-holes mutations[6,7]. Three criteria were used to identify suitable pairs of C_H3 residues for creating an inter-H chain disulfide bond. First, the α-carbons should be separated by distances similar to those found in naturally occurring disulfide bonds (5.0–6.8 Å)[13]. Distances up to 7.6 Å were considered to allow for possible movement of the main chain. Second, the residue pairs should involve a different residue on each C_H3 domain. Third, cysteine replacement of the residues should permit the formation of a disulfide bond with favorable geometry[13].

Six pairs of C_H3 residues satisfy the criteria above and were chosen for cysteine replacement mutations. These six residue pairs are located near the edge of the C_H3 domain interface either proximal (K392C with D399′C and V397C with T394′C) or distal (S354C with L351′C, and S354C, E356C, or E357C with Y349′C) to the C_H2 domain (Fig. 2). In contrast, previously identified knobs-into-holes mutations (T366W/T366′S:L368′A:Y407′V) that enhance heterodimerization[6,7] are located at the center of the C_H3 domain interface, away from the sites targeted for cysteine replacements (Fig. 2). These two engineering strategies were combined in an effort to further enhance H chain heterodimerization.

The cysteine replacement, L351C, was predicted and subsequently found to form disulfide-bonded homodimers as well as heterodimers, thus decreasing its utility. The cysteine mutant pair, V397C/T394′C, led to inefficient disulfide bond formation. The four remaining pairs of cysteine mutants (K392C/D399′C, S354C/Y349′C, E356C/Y349′C, and E357C/Y349′C) were installed in both possible orientations into the phage-optimized knobs-into-holes variant, T366W/T366′S:L368′A:Y407′V (ref. 7).

Enhancing Fc heterodimerization. C_H3 variants were compared with wild-type in their ability to direct the formation of an anti-CD3/CD4-IgG antibody/immunoadhesin hybrid (Ab/IA)[14]. The CD4-IgG and anti-CD3 H chain variants were transiently coexpressed in human embryonic kidney 293 cells, along with the anti-CD3 L chain. The yield of Ab/IA was optimized by varying the ratios of transfected H chain to IA DNA as described previously[6]. The products were affinity-purified using staphylococcal protein A and quantified by SDS-PAGE and scanning laser densitometry[6,7]. Fc heterodimerization is evident from the molecular weight of the resultant Ab/IA hybrid as this is distinct from both IgG and IA homodimer. All Ab/IA variants (v1–v16) were recovered in yields similar to molecules containing the wild-type C_H3 domain.

As with the parent molecules[4,7], each of the six double cysteine replacement mutants (disulfide-C_H3 variants) gave rise to three major products: IgG, Ab/IA hybrid, and IA homodimer (Fig. 3A). However, Ab/IA hybrids from the disulfide-C_H3 variants show increased electrophoretic mobility compared with the Ab/IA constructed using wild-type C_H3 domains, consistent with formation of the designed disulfide bonds (Fig. 3A). Mutagenesis of the hinge cysteines to serine provided direct evidence for formation of the engineered disulfide bond. Covalently bonded Ab/IA hybrids were observed by SDS-PAGE for disulfide-C_H3 variant v11 in which the two hinge cysteines were mutated to serine. In contrast, such covalent hybrids were not observed for molecules with hinge mutations and wild-type C_H3 domains (Fig. 3A). The disulfide variant, v1 (K392C/D399′C), increased the Ab/IA yield over wild-type: 73% versus 51%, respectively (Table 1), whereas the five other disulfide variants constructed (v3 to v7) did not significantly impact Fc heterodimerization. This improvement with variant v1 apparently reflects disulfide bond formation rather than replacement of residues K392 and D399 as the corresponding double serine mutant (K392S/D399′S, v2) gave Ab/IA yields similar to wild-type (Table 1).

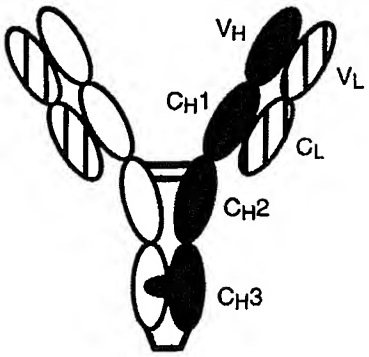

Figure 1. Generic route to bispecific IgG. H chains (filled and unfilled) were remodeled so that they heterodimerize but do not homodimerize using knobs-into-holes C_H3 mutations[4,7] and an engineered disulfide bond (kinked line) between the C_H3 domains. Antibodies that share the same L chain (striped) were chosen to circumvent the problem of L chains pairing with noncognate H chains. Two naturally occurring hinge region disulfide bonds are indicated by horizontal lines.

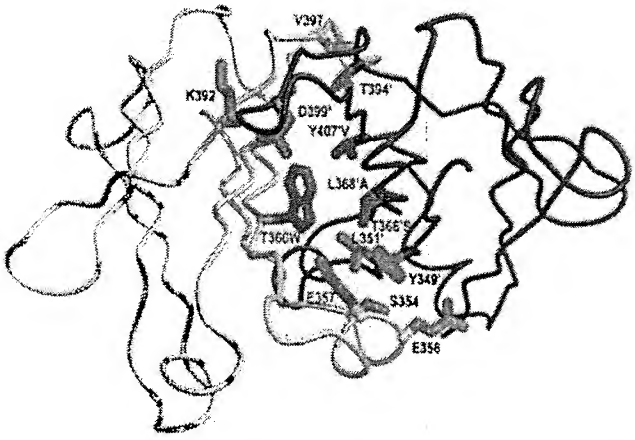

Figure 2. C_H3 residues targeted for cysteine replacement mutations (cyan) highlighted in the 2.9 Å structure of human IgG, Fc (ref. 18). The knobs-into-holes mutations, T366W/T366′S:L368′A:Y407′V (refs. 6 and 7; magenta) were introduced by molecular modeling.

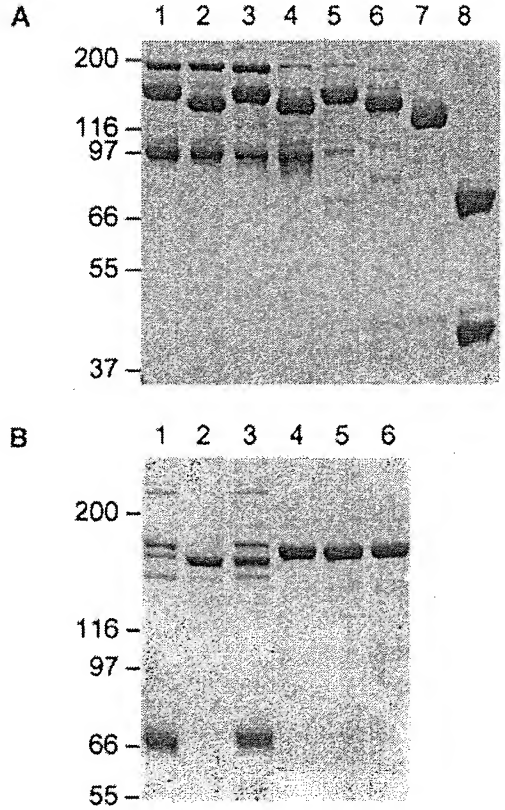

Figure 3. SDS-PAGE analysis of protein A purified products from transiently coexpressed antibody H and L chains and IA. (A) Ab/IA production. Lane 1: wild-type; lane 2: v3; lane 3: v2; lane 4: v1; lane 5: v8; lane 6: v11; lanes 7 and 8: C231S:C234S/C231′S:C234′S hinge mutations in context of v11 and wild-type, respectively. (B) Bispecific IgG production. Lane 1: $H_H + L_{MH}$; lane 2: $H_H + H_M + L_{MH}$; lane 3: $H_M + L_{MH}$, lane 4: $H_{M,wt} + L_{MH}$; lane 5: $H_{H,wt} + H_{M,wt} + L_{MH}$; and lane 6: $H_{M,wt} + L_{MH}$, where H_H and $H_{H,wt}$ correspond to mutant (S354C:T366W) and wild-type versions of the anti-HER3 H chain, H_M and $H_{M,wt}$ correspond to mutant (Y349C:T366S:L368A:Y407V) and wild-type versions of the anti-Mpl H chain, and L_{MH} represents the common L chain.

Table 1. Yields of Ab/IA hybrids from C_H3 variants.

Variant	Mutations		Yield of Ab/IA hybrid (%)
	Ab	IA	
wild-type	none	none	51±1
v1	D399C	K392C	73±3
v2	D399S	K392S	55±1
v3	Y349C	S354C	54±4
v4	Y349C	E356C	55±6
v5	Y349C	E357C	57±4
v6	L351C	S354C	56±3
v7	T394C	V397C	57±2
v8	T366W	T366S:L368A:Y407V	86.7±2.3
v9	T366W:D399C	T366S:L368A:K392C:Y407V	86.5±0.5
v10	T366W:K392C	T366S:D399C:L368A:Y407V	92±1
v11	S354C:T366W	Y349C :T366S:L368A:Y407V	95±2
v12	Y349C:T366W	S354C:T366S:L368A:Y407V	90±1
v13	E356C:T366W	Y349C:T366S:L368A:Y407V	94±2
v14	Y349C:T366W	E356C:T366S:L368A:Y407V	95.5±0.5
v15	E357C:T366W	Y349C :T366S:L368A:Y407V	93±2
v16	Y349C:T366W	E357C:T366S:L368A:Y407V	91.0±1.0

The yield of Ab/IA was estimated by SDS-PAGE followed by scanning laser densitometry. Data are the mean (± SD) from two or more independent experiments. Mutations are denoted by the amino acid residue and number (Eu numbering scheme of Kabat et al.[19]) followed by the replacement amino acid. Multiple mutations are represented by the single mutations separated by a colon.

When evaluated in the context of the phage-optimized C_H3 variant, v8, the cysteine replacement mutations increased the Ab/IA yield up to a maximum of approximately 95% (v11, v13, v14) or left it unchanged (v9) (Table 1). The engineered disulfide bonds exhibit a context-dependent effect upon Fc heterodimerization. For example, the mutations K392C/D399′C are most effective in increasing the Ab/IA yield for an otherwise wild-type C_H3. In contrast, mutations S354C/Y349′C and Y349′C/E356C give the greatest improvement in Ab/IA yield for variant v8. One of the most successful variants, v11 (S354C:T366W/Y349′C:T366′S:L368′A:Y407′V), was utilized in the construction of a BsIgG.

Identification of antibodies using identical L chains. Panning of a large human scFv library[12] identified a panel of 11 antibodies specific for the extracellular domain (ECD) of human epidermal growth factor receptor 3 (HER3) also known as c-*erb*B-3 (ref. 15). The V_H and V_L amino acid sequences of the anti-HER3 scFv were compared with 23 scFv that bind to the human thrombopoietin receptor, c-Mpl (data not shown). Five of the 11 anti-HER3 clones share an identical V_L amino acid sequence with one or more Mpl-binding clones. Conversely, seven out of 23 anti-Mpl scFv share the same V_L as one of the anti-HER3 clones. In contrast to the V_L sequences, the V_H amino acid sequences share an identity level of only 40–90% for any pair of anti-Mpl and anti-HER3 clones.

The frequency of identical L chains of clones derived by panning the phage library of Vaughan et al.[12] was assessed by comparing 117 V_L amino acid sequences for scFv binding 11 different antigens (Table 2). scFv sharing identical L chains were identified for the majority (50/55) of the possible pairwise combinations of two different antigen specificities. Identical L chains were found in all cases except where five or fewer sequences were available for one or both of the antigen specificities. This high frequency of antibodies sharing the identical L chain reflects the very limited size of the L chain repertoire in the phage library. The majority (95/117) of the V_L sequences are represented by just nine clads, each comprising sequences that differ by five amino acid residues or fewer from the consensus sequence of the clad. Our V_L sequence data closely match those reported by Vaughan et al.[12]

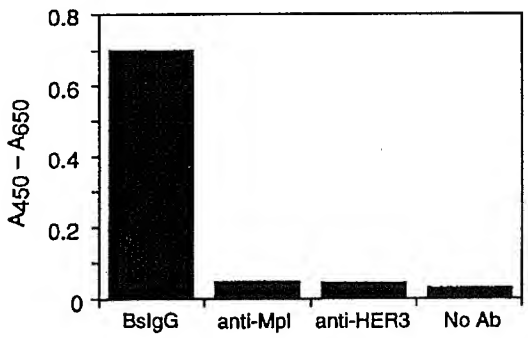

Figure 4. Sandwich ELISA for detection of simultaneous binding to Mpl-IgG and HER3-IgG. Antibodies tested were the anti-Mpl x anti-HER3 BsIgG containing the mutations Y349C:T366S:L368A:Y407V/T366′W:S354′C, and the corresponding parental anti-Mpl or anti-HER3 antibodies with mutated Fc regions.

Efficient construction of bispecific IgG. The anti-Mpl scFv, 12B5 (Genbank accession number, AF048775), and anti-HER3 scFv clone H6 (Genbank accession number, AF048774) use identical V_L sequences and substantially different V_H sequences. These scFv fragments were reformatted as IgG to test the scheme for constructing BsIgG (Fig. 1). The common L chain was cotransfected with the two H chains containing the C_H3 mutations from variant v11. The IgG products were then purified by protein A affinity chromatography and analyzed by SDS-PAGE (Fig. 3B).

The BsIgG preparation gave rise to a single major band showing greater mobility than IgG containing wild-type C_H3 domains (Fig. 3B). This increase in electrophoretic mobility is consistent with the formation of the engineered disulfide bond in the BsIgG. As anticipated, the BsIgG, but not the parental anti-Mpl and anti-HER3 IgG, showed simultaneous binding to Mpl and HER3 ECD antigens in a sandwich ELISA (Fig. 4).

Antibody with C_H3 mutations supports efficient antibody-dependent cell-mediated cytotoxicity. We also investigated the effect of Fc mutations (S354C:T366W and Y349C:T366S:L368A:Y407V) on the ability of an IgG to support antibody-dependent cell-mediated cytotoxicity (ADCC). We chose to make this comparison in the context of a monospecific, humanized anti-HER2 IgG, huMAb4D5-5 (ref. 16), rather than the BsIgG, because the control molecule containing a wild-type Fc region is easily prepared for the monospecific IgG but not for the BsIgG.

Anti-HER2 antibodies containing remodeled or wild-type Fc regions had similar potency in ADCC with the HER2-overexpressing breast cancer cell line SK-BR-3 (Fig. 5). Both antibodies showed comparable, low activity against the nontumor breast epithelial cell line HBL100, which expresses 33-fold less HER2 than SK-BR-3 cells[17] (Fig. 5). This maintenance of ADCC function is consistent with the prediction that the C_H3 mutations are fully buried and do not propagate major structural changes to the surface of the corresponding C_H3 domains.

Table 2. Usage of identical V_L by scFv binding to different target antigens.

First specificity	Total # scFv	Second specificity										
		Axl	GCSF-R	IgE	IgE-R	c-Mpl	MuSK	NpoR	Rse	HER3	Ob-R	VEGF
Axl	12	2	2	0	1	2	1	0	3	2	2	1
GCSF-R	11	2	0	1	2	2	1	2	2	2	3	2
IgE	2	0	1	0	1	1	0	1	1	1	1	0
IgE-R	4	1	2	1	0	1	0	1	2	1	1	1
c-Mpl	23	3	3	1	1	5	5	3	5	7	5	2
MuSK[17]	3	1	1	0	0	3	0	1	1	2	1	1
NpoR	5	0	2	1	1	2	1	0	1	2	2	1
Rse	20	5	3	1	3	8	2	1	7	7	5	2
HER3	11	2	2	1	1	5	2	2	4	3	4	4
Ob-R	18	5	3	1	1	9	1	2	8	9	7	1
VEGF	8	1	3	0	1	2	2	2	1	4	2	2

Values are the frequencies of scFv sharing identical V_L amino acid sequences identified by alignment of 117 V_L sequences for clones obtained using the library of Vaughan et al.[12] Axl: Axl receptor tyrosine kinase; GCSF-R: granulocyte colony simulating factor receptor; IgE-R: IgE receptor; c-Mpl: human thrombopoietin receptor; MuSK: muscle-specific tyrosine kinase receptor; NpoR: Npo receptor; Rse: Rse receptor tyrosine kinase; HER3: human epidermal growth factor receptor 3; Ob-R: leptin receptor; VEGF: vascular endothelial growth factor. All antigens are human in origin except for IgE, which is murine.

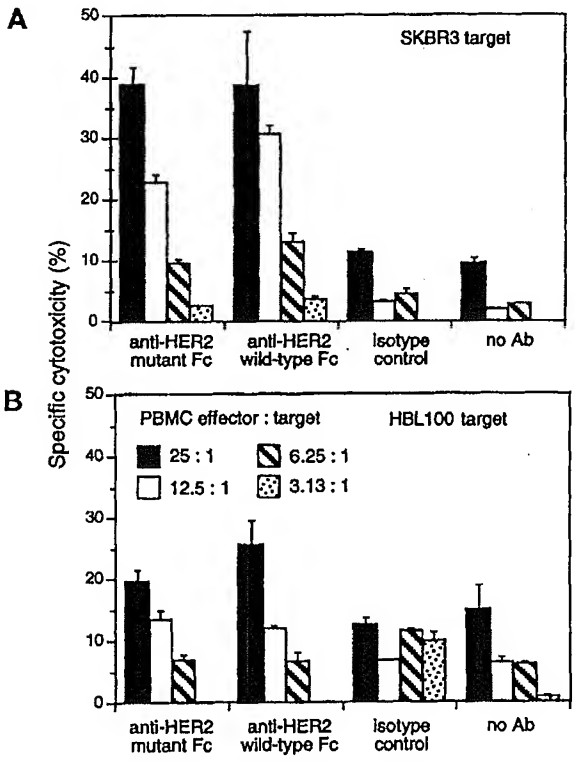

Figure 5. ADCC mediated by huMAb4D5–5 (Ref. 16) containing either a mutant (S354C:T366W/Y349′C:T366′S:L368′A:Y407′V) or wild-type Fc region or an isotype-matched control antibody (E25, Ref. 35) and human peripheral blood mononuclear cell (PBMC) effector cells. Data are the mean of triplicate measurements.

Discussion

We have developed an efficient method for the construction of human BsIgG by remodeling antibody H chains for heterodimerization and using a single L chain to circumvent L chain mispairing (Fig. 1). We anticipate that BsIgG technology will be applicable to antibodies recognizing virtually any pair of antigens, as antibodies with different antigen-binding specificities that share identical L chains have been routinely identified by us (Table 1) and others[11,12]. In addition, the C_H3 mutations required for H chain heterodimerization can be incorporated into H chains of any antigen-binding specificity. These engineered H chains promise to be useful in heterodimerization of other human immunoglobulins because the mutated residues are fully conserved across human IgG isotypes and the majority of the C_H3 interface residues are highly conserved[18–20]. The engineered H chain variants may also be applied directly to the construction of bispecific immunoadhesins[21] in addition to the BsIgG and Ab/IA shown here.

The BsIgG comigrates on SDS-PAGE with one of the five products from either parental IgG (Fig. 3B). The electrophoretic mobilities of the minor contaminants in the BsIgG preparation are consistent with the presence of antibody aggregates and monomers, and three lines of evidence taken together support the notion that our BsIgG preparation contains little contaminating parental IgG. First, the BsIgG contains an Fc region identical to that of variant v11, which yielded 95% of the Ab/IA hybrid. It seems very likely that this same Fc region would be equally effective in directing the heterodimerization of two antibody H chains. Second, contamination of the BsIgG with parental IgG would be expected to give rise to all five of the parental bands rather than just one. Third, the BsIgG preparation binds simultaneously to both antigens, whereas each parental IgG binds only to its respective, cognate antigen.

The potential risk of eliciting an antibody or T cell response is a frequently voiced concern when engineering proteins for human therapy. These immunogenicity issues can only be definitively addressed through clinical trials. Nevertheless, the risk of immunogenicity seems small as the C_H3 mutations are fully buried and few in number (six). Humanized antibodies containing several dozen foreign and mainly exposed residues in their variable domains have elicited undetectable[22–25] or only minor[26,27] antiglobulin responses.

Antigen-binding affinities in the low nanomolar or even subnanomolar range are desirable and may be necessary for each arm of a BsIgG destined for human therapy. In some cases it will likely be possible to construct high-affinity human BsIgG using scFv isolated directly from a large naive phage library. Vaughan et al.[12] have reported many high-affinity scFv (K_d=0.3–8.0 nM) from the same scFv phage library we used in this study to identify antibodies using the same L chains. These high-affinity scFv include several with specificities that have obvious clinical relevance; tumor-associated antigen (CEA), a radionuclide chelator (DPTA), and a chemotherapeutic drug (doxorubicin). Thus, high-affinity antigen binding is possible even with a very limited L chain repertoire. These high affinities presumably reflect the fact that the major determinants contributing to the specificity and energetics of antigen binding reside on the H chain for these phage-derived antibodies.

Sometimes it may be necessary to increase the binding affinity for one or both of the chosen antigens for a BsIgG. For example, in the case of the BsIgG evaluated in this paper, the anti-Mpl binding affinity is low (K_d=96 nM for Fab fragment, data not shown). Affinity maturation of antibody fragments from the nanomolar to picomolar range has been accomplished by mutagenesis of H and L chains and selection using phage display libraries[28,29]. For BsIgG, any mutation of the L chain would have to be evaluated in terms of maintaining binding to the two different antigens.

It may be desirable to construct a BsIgG using the common L chain format (Fig. 1) in conjunction with H and L chains derived from an existing antibody. In this case it will be necessary to identify a second H chain that pairs with the original L chain and binds the second antigen of interest. The Fab phage library and the panning strategy of Figini et al.[30] offer a promising route for identifying such an H chain.

Experimental protocol

Modeling of disulfide bonds. Disulfide bonds were modeled into the human IgG, Fc[14] as described for huMAb4D5-8 Fv (ref. 31) using Insight II release 95.0 (Molecular Simulations, San Diego, CA).

Construction of C_H3 variants. Mutations were introduced into the C_H3 domain of a humanized anti-CD3 H chain or CD4-IgG by site-directed mutagenesis[32]. Mutants were verified by dideoxynucleotide sequencing[33] using Sequenase version 2.0 (United States Biochemicals, Cleveland, OH).

Expression and purification of Ab/IA variants. Human embryonic kidney 293S cells were cotransfected by the calcium phosphate method[34] with a total of 10 μg DNA encoding CD4-IgG plus anti-CD3 L and H chains. The ratio of phagemid DNA for L and H chains was fixed at 3:1 so that the L chain would not be limiting. Additionally, the IA-encoding DNA was used in excess to that for the H chain because the IA is less efficiently expressed than the IgG. The transfected DNA ratios tested ranged from 3:1:3 through 8:1:3 for IA:H chain:L chain. The 293S cells were washed with phosphate-buffered saline (PBS) prior to transfection to remove any residual immunoglobulin from the fetal calf serum used in the media. Following the transfection (72 h) Fc-containing proteins were purified from conditioned media using immobilized protein A (ProSep A, BioProcessing Ltd., Consett, UK) and buffer-exchanged into PBS. Iodoacetamide was added to protein preparations to a final concentration of 50 mM to prevent shuffling of disulfide bonds.

Quantitation of Fc-containing proteins. Samples were electrophoresed in 8% (Ab/IA) or 6% (BsIgG) polyacrylamide gels (Novex, San Diego, CA) and visualized by staining with Serva blue G (Serva, Heidelberg, Germany). Gels

were destained leaving a faint blue background to permit detection and quantitation of minor contaminants. Dried gels were analyzed by scanning laser densitometry (GS-670; Bio-Rad, Hercules, CA) and protein products were quantified with Molecular Analyst software (Bio-Rad).

Identification of scFv by phage library panning. scFv binding HER3 were obtained using a large human scFv phage library[12] (Cambridge Antibody Technology, Melbourne, UK) and panning for three rounds using HER3-IgG (10 μg in 1 ml PBS) coated Immunotubes (Maxisorp; Nunc, Naperville, IL). Panning and phage rescue were then performed as described by Vaughan et al.[12] with the following modifications. A humanized anti-IgE antibody[35] at a concentration of 1 mg/ml was included in each panning step to absorb Fc-binding phage. Clones from rounds two and three of panning were screened by phage and scFv ELISA using the HER3-IgG and also a control IA, CD4-IgG[34]. The diversity of antigen-positive clones was analyzed by PCR-amplification of the scFv insert using the primers, fdtetseq and PUC19 reverse[11] and by digestion with BstNI[37] (ref. 37). Up to five clones per BstNI fingerprint were then cycle-sequenced using fluorescent dideoxy chain terminators (Applied Biosystems, Foster City, CA) using M13 reverse (New England Biolabs, Beverly, MA) and myc seq 10 primers[11]. Samples were analyzed using automated DNA sequencers (models 373 and 377; Applied Biosystems) and sequences analyzed using Sequencher (Gene Codes, Ann Arbor, MI).

Alignment of V_L sequences. The nucleotide sequence data for scFv fragments were translated to derive corresponding protein sequences. The V_L sequences were then compared using the program ALIGN with the algorithm of Feng and Doolittle[38-40] to calculate the percentage identity between all pairwise combinations of chains.

ADCC. Cytoxicity assays were performed with ^{51}Cr-labeled SK-BR-3 and HBL100 target cells and human peripheral blood lymphocytes as effector cells as described[17], except that the lymphocytes were not activated with IL-2. The concentration of antibodies in the ADCC assays was 125 ng/mL.

ELISA. PBS was used as buffer for all steps. Individual wells of a 96 well plate (Maxisorp, Nunc) were coated overnight with Mpl-IgG at 5 μg/ml, washed and then blocked for 1 h with 0.5% (wt/vol) bovine serum albumin. The primary antibodies were the anti-Mpl x anti-HER3 BsIgG containing the mutations Y349C:T366S:L368A:Y407V/T366′W:S354′C and corresponding parental anti-Mpl or anti-HER3 antibodies with mutated Fc regions. The primary antibodies (1 μg/mL) were individually incubated for 2 h at 23°C with biotinylated HER3-IgG and a 1:5000 dilution of streptavidin-horseradish peroxidase conjugate (Boehringer Mannheim, Indianapolis, IN) and then added to the wells and incubated for an additional 1 h at 23°C. Peroxidase activity was detected with TMB reagents as directed by the vendor (Kirkegaard and Perry Laboratories, Gaithersburg, MD).

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